FORM PTO-1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: UNMC 6310Z

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U.S. APPLICATION NO. (If known, see 37 C.F.R.1.5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT US96/16825	18 October 1996	20 October 1995

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN PRODUCING CELLS

APPLICANT(S) FOR DO/EO/US: Sam D. Sanderson, Michael A. Hollingsworth and Richard A. Tempero

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Verified Statement Claim Small Entity Status
Copy of PCT/IB/308 - notice informing applicant of the communication of the international application to the designated offices

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
	PCT US96/16825	UNMC 63102

17. [X] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....		
International preliminary examination fee paid to USPTO (37 CFR 1.482)		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2)) paid to USPTO.....		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 98.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	24 -20 =	4	X 22.00	$ 88.00	
Independent Claims	1 -3 =	0	X 82.00	$ 0	
Multiple dependent claims(s) (if applicable)			+270.00	$ 0	
TOTAL OF ABOVE CALCULATIONS =				$ 186.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$ 93.00	-
SUBTOTAL =				$ 93.00	
Processing fee of **$130.00** for furnishing the English translation later the [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
TOTAL NATIONAL FEE =				$ 93.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$ 0	
TOTAL FEES ENCLOSED =				$ 93.00	
				Amount to be: refunded	$
				charged	$

a. [X] A check in the amount of $ 93.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-1406. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

SIGNATURE

Janet E. Reed, Ph.D.

NAME

36,252

REGISTRATION NUMBER

COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS

Pursuant to 35 U.S.C. §202(c), it is hereby acknowledged that the U.S. Government has certain rights in the invention described herein, which was made, in part, with funds from the National Institutes of Health, grant numbers CA 57362 and CA 36727.

The disclosure of commonly-owned, co-pending U.S. Application Serial No. 08/299,285, is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the field of vaccines and stimulation of acquired immunity. In particular, the present invention provides novel compositions designed to deliver specific antigens to antigen presenting cells and simultaneously deliver signals to those cells that produce a desired immune response.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parenthesis in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

The basis of acquired, specific immunity in an organism is the ability to discriminate between self and non-self antigenic substances. The mammalian immune system uses cell surface molecules known as the major histocompatibility complex (MHC) for discriminating between self from non-self. There are two classes of MHC molecules: Class I molecules are found on all nucleated

cell types in the body; Class II molecules are found mainly on cells involved in producing immune responses. Most specific immune responses are generated against peptides or peptide derivatives associated with MHC molecules.

The structure of MHC molecules is such that they naturally bind small peptides, glycopeptides, phosphopeptides, and the like. One important function of MHC molecules is to bind peptides that are derived from processed products of proteins expressed in cells expressing the MHC molecules, and to transport these to the cell surface for display to the immune system. In this way, some MHC molecules function to expose the immune system to peptides that are representative of normal cellular proteins. This process occurs during development, when self is learned, and continues throughout the organism's lifespan. Different mechanisms of immune tolerance prevent the organism from responding to "self" peptides associated with MHC.

The introduction of non-self proteins into cells results in the appearance of new and different peptides in association with the MHC molecules; these are recognized as "non-self," resulting in an immune response. For example, viral infection of a cell will result in the production of viral peptides expressed on the surface of antigen-presenting cells in association with MHC molecules (generally Class I MHC). Viral peptides presented with MHC molecules at the cell surface will often be recognized as foreign and an immune response will be mounted. Autoimmune disease can occur if tolerance to some self peptides is lost, or if immune response is produced against viral or other foreign proteins that cross react with normal peptides in the host organism.

In the case of bacterial infections or other insults from external sources, new proteins or compounds enter the organism. Some cells involved in the immune

response are capable of phagocytosing foreign organisms or proteins. These immune cells degrade (process) the protein products and the derived peptides are expressed at the cell surface in association with MHC molecules, where a specific adaptive immune response is generated against novel non-self components. This activity is called antigen processing and presentation and cells that mediate this activity are called Antigen Presenting Cells (APC's). Many different immune cell types, including macrophages, dendritic cells, B cells, and other associated cell types, perform this function.

Antigen alone is often insufficient to produce an immune response. Sometimes, antigen must be presented with accompanying "signals" that are mediated by ligand-receptor interactions between the APCs and the responding lymphocytes or between these cells and soluble factors that are present in the surrounding environment. The soluble factors include cytokines and other mediators of inflammation that are usually present at sites of infections or insult (complement, kinins, other growth and cytokine factors). The signals can be positive in nature, resulting in lymphocyte proliferation and generation of an adaptive immune response, or negative in nature, resulting in apoptosis of responding lymphocytes and perhaps immune tolerance to that antigen. Antigen presentation often occurs in the presence of helper T cells or other cell types that secrete arrays of cytokines, which influence or determine the type of immune response that is induced. At a cellular level, specific immune responses are generated in a mixed cellular environment that includes different types of antigen presenting cells, helper T lymphocytes, other types of regulatory cells, and the responding lymphocytes (B cells for antibody responses and T cells for cellular responses). Direct recognition of peptides by T cells can also occur with some cell types, such as allografts, where the allogeneic MHC is directly recognized as

foreign.

Antigen processing and its impact on types of immune responses to specific antigens. The mechanism by which antigen is processed and presented and the parameters that determine the types of immune responses that are generated (antibody versus cellular) are at present not well understood for many antigens. It is believed that there are different classes of APCs that can produce different types of immune responses. In general, APC-induced responses to exogenous antigens that are taken up by endocytosis are believed to be presented to the immune system in the context of Class II MHC and lead to recruitment of T helper cells that interact with B cells and ultimately produce an antibody response. In contrast, endogenous peptides from cells associate with MHC Class I molecules and produce cellular activities that include cytotoxic T lymphocytes (CTL) and Delayed Type Hypersensitivity (DTH) T-cells. There are important exceptions to these mechanisms. For example, many CTLs reactive with exogenous peptides have been described, and it is possible to generate CTLs to specific peptides that have been added to *in vitro* cultures of immune cells.

Other factors can determine the types of immune responses that are generated. For example, the nature of peptide association with MHC (either Class I or Class II) is an important factor that influences types of immune responses. In the case of Class I MHC molecules, there are specific binding motifs for peptide association (Rammensee et al, Ann. Rev. Imm. 11: 213, 1993). Binding motifs have been established for H-2 K^d, K^k, D^d, and other murine and human MHC. There are also parameters of peptide sequence that determine affinity for class II MHC. Thus, the types of peptides to which an individual can mount an immune system response are in part determined by the immunogenetic genotype and phenotype, which establish the shape and structure of the MHC

molecules expressed by that individual.

In summary, the types of immune response that are generated in an organism in response to antigenic challenge is the result of a myriad of contributing factors, including: the immunogenetic background of the individual, prior sensitization to antigens, the route and form of antigen exposure, age and gender of the organism, and other factors. Almost all acquired immune responses that involve specific T-cell recognition are directed toward small peptides bound to the peptide binding groove of MHC molecules, the obvious exception being the response to superantigens. Cellular immune reaction (T-helper reaction, CTL, DTH) to peptides bound to MHC are usually generated through presentation of the antigen to T cells by antigen-presenting cells (APCs).

Tumor Vaccines. Cancer cells express aberrant molecules known as tumor-associated antigens. The immune system has the potential to recognize such structures as "foreign" and to mount specific immune responses against them, so as to reject tumor cells in much the same way that an allograft is rejected. This provides the basis for interest in the development of active specific immunotherapeutic (ASI) agents (cancer "vaccines") based on cancer-associated antigens.

Early studies on rodent tumors induced by chemical carcinogens, ultraviolet radiation, or viruses showed induction of immunological rejection of secondary tumor challenge. Subsequent studies on spontaneous tumors showed that these animals were incapable of inducing immune-mediated rejection of the tumor. Although a large number of human tumor-associated antigens have been characterized, most of these are also expressed by some normal cells. Therefore, immunological tolerance to such molecules makes it difficult to stimulate responses against such antigens. Moreover, it is a concern that induction of strong immune responses

against self molecules may result in the development of autoimmune disorders. Since tumor-specific antigens are seldom detected in spontaneous cancers, approaches to develop active specific immunotherapy for common cancers, based on tumor-associated antigens, have been viewed with pessimism.

Nonetheless, interest in tumor immunology and the development of ASI in particular has persisted. There are at least four reasons for the current interest in ASI approaches. First, cell-mediated immune responses have been recognized as the key factor in immunological rejection of cancer. T cells recognize processed peptides in association with major histocompatibility complex (MHC) molecules, so intracellular proteins can give rise to peptide targets for cell-mediated responses. Further, since antigen processing and presentation are critical steps in T cell recognition, cancer-associated alterations (in its post-translational processing or levels of expression) of a self protein may result in presentation of novel peptide fragments on cancer cells. Secondly, tumor specific point mutations in certain genes have been characterized in several animal and human cancers. Some of these mutations generate novel peptide fragments that bind MHC molecules resulting in the production of new epitopes for recognition by T cells. This process allows for the induction of specific immune responses against cancer cells carrying such mutations. Third, manipulation of immune responses using cytokines, mutated antigens, and other means have sometimes resulted in tumor rejection even in cases of tumors that express weakly immunogenic antigens. Fourth, some individuals with severe immunodeficiencies have a higher incidence of tumors than the normal population, suggesting that the immune system plays an important role in eliminating some tumors.

Various methods have been utilized for stimulating general immune responses, especially for non-

antigenic or weakly antigenic substances of interest. For example, adjuvants, such as complete Freund's and Ribi's, have long been used for this purpose. These adjuvants comprise oily solutions containing components, such as lipopolysaccharides that stimulate generalized immune responses. It is believed that the oils surround a water-soluble antigen, such as a peptide, thereby protecting it from degradation in the body and facilitating phagocytosis and passage through cell membranes of antigen presenting cells.

Another approach to stimulating the immunogenicity of a weakly-antigenic peptide or protein has been to couple the weak antigen to a carrier protein that is known to be a good immunogen. Common carrier proteins include keyhole limpet hemocyanin, fowl gamma-globulin and bovine serum albumin. Alternatively, the immunogencity of a weak antigen may be enhanced by polymerizing it into large aggregates by way of cross-linking agents, such as glutaraldehyde. Both these methods rest on the notion that a weak antigen coupled to a strong antigen will enhance the generalized immune response. In a similar method, solid-phase resins and peptide synthetic methods may be employed to synthesize a peptide repeatedly, to form a highly-branched complex. Again, the basis for this approach is to present the antigen in very unusual (and very "non-self") context to the immune system, to stimulate antibody production.

In yet another approach, a weakly antigenic protein or peptide is attached to a solid particle such as a latex bead or resin. The purpose of this approach is to enhance phagocytosis of the antigen by macrophages. Additionally, peptides and proteins have been encapsulated in liposomes to enhance passage through membranes of antigen presenting cells, to enhance phagocytosis and to stimulate generalized immune responses because of the "non-self" characteristics of the liposome carrier.

The approaches described above have met with varying degrees of success in stimulating the immunogenicity of weakly antigenic or non-antigenic substances. However, they provide only a generalized stimulation of immunity, and are not designed to target specific populations of immune system cells (such as antigen presenting cells). A desired objective in effecting therapeutic intervention in various disease states is to provide a means for specifically targeting a protein or peptide to a population of antigen-presenting cells and thereby stimulate those cells to internalize the antigen of interest and present it to the immune system in an effective, specific context. Insofar as it is known, such a system is not currently available.

SUMMARY OF THE INVENTION

The present invention provides novel compositions and methods for delivering specific antigens to antigen-presenting cells, and simultaneously delivering signals to those cells that produce a desired immune response. The compositions of the invention are sometimes referred to herein as "APC-targeted activating antigens."

According to one aspect of the invention, these APC-targeted activating antigens, which elicit an immune response mediated by an antigen-presenting cell, comprise at least one antigenic moiety functionally linked to at least one targeting moiety that binds specifically to a characteristic determinant on the antigen-presenting cell. For purposes of the present invention, the term "functionally linked" is defined generally as linking of the moieties in such a way that each moiety retains its intended function. This is particularly relevant with respect to the targeting moiety, which is designed to bind to a characteristic determinant on the antigen-presenting cell.

Antigen-presenting cells contemplated for

targeting according to the present invention include, but are not limited to, monocytes, dendritic cells, macrophages, B cells and some T cells. In preferred embodiments of the invention, the characteristic determinant on the selected APC is a cell surface receptor and the targeting moiety of the APC-targeted antigen is a ligand that binds to the receptor. It is particularly preferred that the cell surface receptor be an immunomodulatory receptor. Suitable cell surface receptors include, but are not limited to, C5a receptor, IFNγ receptor, CD21 (C3d) receptor, CD64 (FcγRI) receptor, and CD23 (FcεRII) receptor.

One exemplary APC-targeted antigen of the invention is designed to bind to the C5a receptor, and the targeting moiety is a C5a receptor ligand, which is preferably a peptide analog of C5a corresponding to the C-terminal 10 residues of C5a. Another exemplary composition of the present invention is designed to bind to the IFNγ receptor, and comprises a targeting moiety which is a IFNγ receptor ligand, preferably a peptide analog of IFNγ corresponding to the N-terminal 39 residues of IFNγ.

The antigenic moiety of the APC-targeted antigens of the invention can comprise essentially any antigenic substance, including, but not limited to, peptides and proteins, glycopeptides and glycoproteins, phosphopeptides and phosphoproteins, lipopeptides and lipoproteins, carbohydrates, nucleic acids and lipids. The APC-targeted antigens can comprise more than one antigenic moiety, and likewise can comprise more than one targeting moiety. Moreover, these moieties can be functionally linked in several fashions. For instance, if "T" represents a targeting moiety, and "Ag" represents an antigenic moiety, the APC-targeted antigens of the present invention may be oriented as follows:

Ag - T;

T - Ag;

T_1 - Ag - T_2;

T_1 - $[Ag]_n$ - T_2 (wherein $[Ag]_n$ represents a multiplicity of antigens.

Examples of the general formulas set forth above include:

Ag - C5a agonist peptide;

IFNγ peptide - Ag;

IFNγ peptide - $[Ag]_n$ - C5a agonist peptide.

According to other aspects of the present invention, methods are provided for using the APC-targeted antigens of the invention. These include methods of activating an antigen-presenting cell with a targeting ligand and methods of eliciting an antigen presenting cell-mediated immune response in a subject in which such a response is desired. General methods of immunizing or vaccinating a patient requiring such treatment, methods of treating a tumor, and methods for producing antibodies specific for a pre-determined antigen for use as research tools or for diagnostic purposes are also contemplated to be within the scope of the present invention.

The numerous features and advantages of the compositions and methods of the present invention are described more fully in the detailed description set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the antibody titer produced in mice immunized with the indicated peptide constructs, as determined by radioimmunoassay, and shows the relationship between the amount of ^{125}I-goat anti-mouse antibody bound vs. the dilution factor of mouse sera which had been incubated in microtiter wells coated with the MUC1 epitope peptide.

Figure 2 is a graph illustrating the increase in

antibody titer in the sera of mice collected either before (pre) or after immunization with peptides 3 (YKQGGFLGLYSFKPMPLaR) (SEQ ID NO:2) and 4 (YSFKPMPLaRKQGGFLGL) (SEQ ID NO:5) as determined by radioimmunoassay and shows the relationship between the amount of ^{125}I-goat anti-mouse antibody bound and the dilution factor of mouse sera which had been incubated in microtiter wells coated with MUC1 epitope peptide. Note that peptides 3 and 4 comprise two moieties, a targeting ligand and an antigen to which an immune response is desired.

Figure 3 is a graph illustrating the titers of antibody classes and subclasses produced in mice following immunization with peptide 3 (YKQGGFLGLYSFKPMPLaR) (SEQ ID NO:2) as determined by ELISA using rabbit anti-mouse IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM, followed by goat anti-rabbit conjugated to peroxidase and detected using p-nitrophenyl phosphate cleavage monitored at 405 nm.

Figure 4 is a graph illustrating the specificity of binding of the antibody subclasses in sera from mice immunized with peptide 3 (YKQGGFLGLYSFKPMPLaR) (SEQ ID NO:2) as determined by ELISA using binding to microtiter wells coated with MUC1 epitope peptide and detection with rabbit anti-mouse IgG2a, IgG2b, or IgM followed by incubation with goat anti-rabbit conjugated to peroxidase and detected using p-nitrophenyl phosphate cleavage monitored at 405 nm.

DETAILED DESCRIPTION OF THE INVENTION

A major obstacle in the development of vaccines and other immunostimulatory agents is the inability of some antigens to be readily taken up and processed by antigen presenting cells. Uptake of antigens by APCs is an essential step for stimulating an effective immune response, since the immune system recognizes the antigen

only after it has been processed by the APC and presented on the surface of the APC in conjunction with the major histocompatibility complex (MHC).

It is known that APCs, including dendritic cells, monocytes, macrophages and B cells, possess functional receptors for numerous molecules that modulate the immune response. It has now been discovered in accordance with the present invention that ligands which bind to these receptors can be conjugated to weakly immunogenic antigens for example, as a way of delivering antigens to the antigen presenting pathway of the APC and simultaneously activating the antigen presenting capacity of the APC. Thus, these conjugates bind to a receptor on the APC surface, transduce a biological signal, and are internalized by the APC. The antigenic moiety of the conjugate is thereby delivered to the antigen presenting pathway of the APC along with the simultaneous activation of the APC.

The above-described conjugates are sometimes referred to herein as "molecular adjuvants" or "APC-targeted activating antigens." The APC-targeted activating antigens of the invention are designed to elicit immune responses mediated by one or more types of antigen presenting cells. Accordingly, an APC-targeted activating antigen comprises at least one antigenic moiety linked to a targeting and activating moiety that binds specifically to at least one characteristic determinant on the selected antigen presenting cell type. This binding is followed by internalization of the APC-targeted antigen and results in presentation of the antigen moiety on the surface of the APC. For purposes of the present invention, the term "antigenic moiety" may refer to any substance to which it is desired that an immune response be produced. The selected antigenic moiety may or may not be capable of eliciting an immune response by conventional means.

The term "determinant" is used herein in its

broad sense to denote an element that identifies or determines the nature of something. When used in reference to an antigen presenting cell, "determinant" means that site on the antigen presenting cell which is involved in specific binding by the targeting ligand moiety of the molecular adjuvant of the invention.

The expression "characteristic determinant" as used herein, signifies an epitope (or group of epitopes) that serves to identify a particular population of antigen presenting cells and distinguish it from other antigen presenting cell populations. Cell-associated determinants include, for example, components of the cell membrane, such as membrane-bound proteins or glycoproteins, including cell surface antigens, histo-compatibility antigens or membrane receptors.

The expression "specific binding", as used herein refers to the interaction between the targeting ligand moiety and a characteristic determinant on the antigen presenting cell population sought to be activated in accordance with this invention, to the substantial exclusion of determinants present on other cells.

Certain exemplary compositions of the invention have been synthesized, and have been shown to elicit APC-mediated immune responses in accordance with the mechanisms described above. For example, antigenic epitopes have been conjugated to the amino-terminal end of a C5a decapeptide agonist capable of binding to C5a receptors present on the surface of many APCs. Mice that were inoculated with an epitope of human MUC1 (a cell surface-associated mucin) conjugated to such a C5a agonist exhibited pronounced antibody titers against the MUC1 epitope, including high titers of specific antibodies with isotypes IgG2a and IgG2b. Mice that were inoculated with (1) MUC1 epitope alone, (2) C5a agonist alone, (3) unconjugated MUC1 epitope and C5a agonist together, or (4) C5a agonist conjugated to MUC1 epitope in a manner in which the biological activity of the C5a

moiety was blocked, did not express a significant specific immune response. These results are described in greater detail in Example 1. Similar results were observed with conjugates of C5a agonist to a 12 kDa polypeptide, serum amyloid A (SAA), as described in greater detail in Example 2. These data tend to demonstrate the feasibility of the invention, which is to use receptor-binding ligands as a way to deliver antigens to APCs, with the simultaneous activation of APCs by the ligand moiety.

As described in greater detail below, the C5a receptor is only one of many receptors expressed on APCs. This invention encompasses the use of various ligands with selectivity to other receptors that mediate signal transduction events in the APCs, to be used alone or in conjunction with C5a agonists to influence the nature of immune response generated, i.e., humoral, cellular, Th1, Th2, and the like. Vaccines and other immunotherapeutic agents can be prepared with an array of such targeting moieties that serve to target the antigen moiety to a specific population of APCs and simultaneously activate these and other cells involved in various immune modulatory pathways.

The detailed description below sets forth preferred embodiments for making and using the targeted antigens of the present invention. To the extent that specific compounds and reagents are mentioned, these are for the purposes of illustration, and are not intended to limit the invention. Any biochemical, molecular or recombinant DNA techniques not specifically described are carried out by standard methods, as generally set forth for example, in Ausubel et al., "Current Protocols in Molecular Biology," John Wiley & Sons, Inc., 1995.

I. Preparing APC-Targeted Activating Antigens

A. Selection of Components

Antigen presenting cells have various receptors

on their surfaces for known ligands. Binding of ligands to these receptors results in signal transduction events that stimulate immune or tolerance responses. Many of these receptors are known to internalize and recycle in the cell. Others are suspected of doing the same. As such, these receptors are ideal targets for delivering antigens and activation signals simultaneously to APCs.

As discussed above, APCs include several cell types including macrophages, monocytes, dendritic cells, B cells, some T cells and other poorly characterized cell types. It is believed that these different classes of APCs can produce different types of immune responses. Accordingly, by targeting a receptor prevalent on a specific population of APCs, a particular desired immune response may be favored. An exemplary list of receptors contemplated for targeting in the present invention, and the rationale for their selection, is set forth below. These APC receptors are particularly appropriate for use in the present invention based on the following criteria: they are receptors expressed on APCs; the receptors are internalized upon binding of ligand; the receptors can transmit signals in the cells that influence antigen processing and presentation by these cells; some of the receptors are believed to be involved in signaling Th1 type cellular responses, whereas others are predicted to generate Th2 type humoral responses. The list set forth below is not exhaustive, but merely representative of the type of targeted receptors preferred in practicing the present invention. Other receptors, or other cell-surface characteristic determinants on antigen presenting cells may also be used as targets for the targeted antigens of the present invention. The receptor or other characteristic determinant need not be directly involved in the immune response.

C5a receptor. This receptor is preferred for use according to the present invention. It is present on PMNs, macrophages, dendritic cells, smooth muscle cells

and some mast cells. A number of biological activities have been ascribed to C5a, mostly associated with inflammatory and immune responses. According to a preferred embodiment, this invention relies on the capability of C5a, as a targeting ligand, to specifically bind to its cognate receptor, so as to activate antigen presenting cells, including macrophages, monocytes and dendritic cells, through a G protein-mediated signal transduction pathway. Subsequent to signal transduction, the receptor/ligand complex is internalized. In the case of dendritic cells, C5a has been shown to induce a Th1 type response.

IFNγ receptor. The interferon γ receptor is expressed on macrophages, monocytes, dendritic cells, other APCs, some B cells, fibroblasts, epithelial cells, endothelium, and colon carcinoma cells. IFNγ binding to its receptor induces macrophage and dendritic cell activation, B cell differentiation, and expression of MHC class I and class II molecules in many cell types. The receptor is involved in signal transduction pathways. IFNγ is mainly produced in the body by activated T cells, particularly during the generation of Th1 type response. It is also produced by CD8+ cytotoxic T lymphocytes following recognition of antigen associated with MHC class I and by natural killer cells stimulated with TNFα and microbial products (**Barclay et al. 1993,**).

CD 21 (C3d receptor). CD 21 is the receptor for the C3d complement fragment. It is a receptor for the Epstein-Barr virus and may be an important interferon α receptor (**Barclay et al.,** ***supra***). CD 21 is expressed on B cells, follicular dendritic cells, other APCs, pharyngeal and cervical epithelial cells, and some thymocytes. It is involved in activation and proliferation of B cells through a signal transduction mechanism and it has been associated with increases in antigen presentation activities by those cells.

CD 64 (FcγRI receptor). CD 64 is a high

affinity receptor for IgG, the only known receptor that binds monomeric IgG (**Barclay et al, *supra***). This receptor is found on macrophages, monocytes and other immune cell populations treated with IFNγ. The IgG_1 binding site resides in the CH2 domain. IFNγ strongly upregulates expression of this receptor, which is the primary receptor involved in antibody-dependent cell mediated cytotoxicity reaction, and phagocytic activity by these cells.

CD 23 (FcεRII receptor). CD 23 is a low affinity receptor for IgE (not related to the high affinity IgE receptor found on basophils and mast cells). It is found on some B cell populations, macrophages, eosinophils, platelets, and dendritic cells (Barclay et al, *supra*). CD 23 mediates IgE dependent cell mediated cytotoxicity and phagocytosis by macrophages and eosinophils, and binding of IgE immunocomplexes increases the efficiency of antigen processing and presentation by some APCs, through a signal transduction mechanism that includes the p59 fyn tyrosine kinase. The ligand for CD 23 is the Cε3 domain of IgE.

As mentioned above, the APC-targeted antigens of the present invention comprise at least one antigenic moiety and at least one targeting moiety. The targeting moiety can be derived from naturally-occurring ligands for a selected receptor on an APC, or analogs and derivatives of such ligands. For instance, the C5a receptor is a preferred receptor for use in practicing the present invention. Naturally-occurring C5a can be utilized as the targeting moiety in the APC targeted activating antigens of the invention. However, native C5a is not preferred for use as the targeting moiety as it induces a myriad of pro-inflammatory responses which may have undesirable side effects. In particularly preferred embodiments of the invention, C-terminal C5a agonist analogs capable of C5a receptor binding and signal transduction in a response selective manner are

utilized. Such analogs are described in detail in commonly-owned U.S. Application Serial No. 08/299,285, the entire disclosure of which is incorporated by reference herein.

An exemplary C5a C-terminal decapeptide agonist preferred for use in the present invention is:

YSFKPMPLaR (SEQ ID NO:1)

This decapeptide is a potent agonist of naturally occurring C5a, and is preferred to naturally occurring C5a because its small size contributes to ease of synthesis and solubility. Moreover, these conformationally biased peptides are stable toward serum carboxypeptidase digestion, express a level biological selectivity, and have been shown to be non-toxic in high concentrations in athymic mice.

Peptide analogs of naturally-occurring interferon γ are also contemplated for use in the present invention. Peptides corresponding to the amino terminal 39 amino acids of IFNγ have been shown to compete for binding with native IFNγ. Antibodies against this domain block biological activity, and removal of the first 10 amino terminal residues eliminates biological activity. This suggests that binding of IFNγ to its cognate receptor is mediated by this portion of the molecule. Accordingly, peptides based on this domain are contemplated to be of use for delivering antigens to APCs expressing IFNγ receptors. In this regard, it should be noted that human and mouse IFNγ are absolutely species specific in binding and activity. Consequently, for stimulating APC-mediated immune responses in mice, the mouse peptides will be utilized, and the human peptide will likewise be utilized for stimulating APC-mediated immune responses in humans. The mouse IFNγ 39 amino acid peptide analog is composed of the following sequence:

HGTVIESLESLNNYFNFFGIDVEEKSLFLDIWRNWQKDG (SEQ ID NO:3)

The human IFNγ 39 amino acid peptide analog is composed of the following sequence:

QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEE (SEQ ID NO:4)

Another ligand contemplated for use in the present invention is the C3dG component of complement. This component is a 348 residue fragment derived by proteolytic cleavage from the C3b precursor (residue 955-1303 of C3; Swissprot accession p01024). C3dG can be converted to C3d (residues 1002-1303) and C3g (residues 955-1001). C3dG and C3d remain associated with non-activator surfaces and serve as opsonins for phagocytosis by macrophages and other antigen presenting cells. Cd 21 is the C3dG and C3d receptor.

The above-listed ligands exemplify the type of ligand preferred for practice of the present invention. However, it will be appreciated by those skilled in the art that other ligands may be utilized as the targeting moiety of the APC-targeted antigens of the invention. These include ligands that are already known in the art, as well as ligands that may be discovered and developed henceforth. Antibodies or antibody fragments also may be used to target APC-specific cell surface antigens.

The type of antigen that can be chosen as the antigenic moiety in the present invention can be any peptide, polypeptide or derivative thereof for which an immune response or antibody production is desired. These include but are not limited to, peptides, polypeptides (i.e. proteins) and derivatives thereof, such as glycopeptides, phosphopeptides and the like. Synthetic peptide and polypeptide derivatives or analogs, or any other similar compound that can be conjugated to a receptor-targeting moiety can be used in the present invention. Moreover, these peptides, proteins and derivatives may comprise single epitopes or multiple epitopes for generating different types of immune responses. Indeed, if an entire protein is conjugated to a targeting moiety, this protein is likely to comprise numerous epitopes, which may vary depending upon the solvent conditions and their effect on secondary and

tertiary structure of the protein. Carbohydrates, nucleic acids and other non-protein substances also may be used as the antigenic moiety. Methods are available in the art for conjugating these substances to the peptide or protein targeting moiety.

In preferred embodiments of the invention, the antigenic moiety comprises agents that are weakly antigenic or non-antigenic under currently available immunization conditions. Many tumor-associated antigens fall into this category, because the antigens also are expressed by normal cells. Therefore, immunological tolerance to such molecules makes it difficult to stimulate responses against such antigens. Other proteins that fall into this category include naturally occurring proteins from one species (e.g., human) for which it would be desirable to produce antibodies in another species but which are recalcitrant to antibody generation in the other species.

One well-characterized tumor antigen is a cell surface-associated mucin that is highly overexpressed and differentially glycosylated by different adenocarcinomas, including breast, pancreas, lung and prostate carcinomas. Aberrant glycosylation of MUC1 by adenocarcinomas results in the addition of some blood group carbohydrate antigens to this core protein and the exposure of epitopes which have been detected by monoclonal antibodies on the core protein that are not exposed on forms of this protein produced by normal epithelial cells. A full-length cDNA sequence of human mucin-1 (MUC1) revealed an encoded protein with an average length of approximately 1200 amino acids (depending on the length of the tandem repeat allele) with several obvious domains: an amino terminal signal peptide; a large domain made up of multiple identical 20 amino acid tandem repeats flanked by several repeats that contain degenerate sequences; a hydrophobic-spanning domain of 31 amino acids; and a cytoplasmic domain of 69 amino acids at the carboxyl terminus. The

most well-characterized tumor associated epitopes described to date for MUC1 are found in the tandem repeat domain. These include carbohydrate structures and protein structures. MUC1 tumor associated epitopes are well characterized, and thus have been proposed to be used for the production of tumor vaccines using conventional methods. Exemplary compositions of the present invention comprise MUC1 epitopes, such as those set forth below, as the antigenic moiety of the APC-1 targeted antigens of the invention, to demonstrate the potential of the present invention as potent tumor vaccines.

MUC1 epitope predicted to bind to class I molecules of the H-2k^b type has sequence homology to the juxtamembrane region of MUC1;

YKQGGFLGL (SEQ ID NO:6)

MUC1 tandem repeat has the sequence:

GVTSAPDTRRAPGSTAPPAH (SEQ ID NO:7)

The components comprising the APC-targeted antigens of the invention can be made separately, then conjugated. For example, a small peptide analog, such as the above-described C5a agonists, may be produced by peptide synthetic methods, and conjugated to a protein which has been purified from naturally occurring biological sources. Alternatively proteins engineered for expression via recombinant methods may be used. Additionally, targeted antigens comprising peptide components (i.e., a peptide antigenic epitope conjugated to a peptide receptor ligand) can be synthesized in tandem by peptide synthetic chemistry according to known methods and as described in greater detail below. Finally, targeted antigens of the invention comprising two larger polypeptide moieties (i.e., a large polypeptide antigen linked to a large ligand) can be made by recombinant techniques. For example, DNA molecules encoding both components can be ligated together by

recombinant means, then expressed as the conjugated fusion protein. Methods of making these different types of compositions are set forth in greater detail below.

B. Peptides

Oligopeptides required for the present invention may be prepared by various synthetic methods of peptide synthesis via condensation of one or more amino acid residues, in accordance with conventional peptide synthesis methods. Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art. When solid-phase synthesis is utilized, the C-terminal amino acid is linked to an insoluble resin support that can produce a detachable bond by reacting with a carboxyl group in a C-terminal amino acid. One preferred insoluble resin support is *p*-hydroxymethylphenoxymethyl polystyrene (HMP) resin. Other useful resins include, but are not limited to: phenylacetamidomethyl (PAM) resins for synthesis of some N-methyl-containing peptides (this resin is used with the Boc method of solid phase synthesis; and MBHA (p-methylbenzhydrylamine) resins for producing peptides having C-terminal amide groups.

During the course of peptide synthesis, branched chain amino and carboxyl groups may be protected/deprotected as needed, using commonly-known protecting groups. In a preferred embodiment, N^{α}-amino groups are protected with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group or *t*-butyloxycarbonyl (Boc groups). Side-chain functional groups consistent with Fmoc synthesis may be protected with the indicated protecting groups as follows:

arginine (2,2,5,7,8-pentamethylchroman-6-sulfonyl); asparagine (O-t-butyl ester); cysteine glutamine and histidine (trityl); lysine (*t*-butyloxycarbonyl); serine and tyrosine (*t*-butyl). Modification utilizing alternative protecting groups for peptides and peptide derivatives will be apparent to those of skill in the art.

C. Proteins

Full-length proteins for use in the present invention may be prepared in a variety of ways, according to known methods. Proteins may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by various methods such as gel filtration, ion exchange chromatography, reverse-phase HPLC and immunoaffinity purification, among others. However, due to the often limited amount of a protein present in a sample at any given time, conventional purification techniques are not preferred in the present invention.

The availability of nucleic acids molecules encoding a protein enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred embodiment, a selected peptide or protein may be produced by expression in a suitable procaryotic or eucaryotic system. For example, a DNA molecule, encoding a peptide or protein component of the invention, or an entire composite targeted antigen of the invention, may be inserted into a plasmid vector adapted for expression in

a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

A peptide or protein produced by gene expression in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, so as to be readily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used for isolating peptides and proteins.

D. Linking Separately-Made Proteins and/or Peptides

In an alternative embodiment, protein and/or peptide components of the invention are synthesized separately, then conjugated using standard methods known by those skilled in the art. For example, a synthetic peptide may be chemically coupled to a protein using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBF). This reagent cross-links amino- and carboxy-terminal thiol groups in the peptide with lysine side chains present in the protein. Alternatively, a synthetic peptide may be coupled to a protein using glutaraldehyde, a common cross-linking agent. Another method for chemically coupling a peptide to a protein is through the use of carbodiimide and 1-(3-dimethylaminopropyl)-3-

ethylcarbodiimide methiodide (EDC). As described in greater detail in Example 2, this method was used to conjugate a C5a C-terminal decapeptide analog to serum amyloid A (SAA). Methods for joining two proteins together are also available.

The peptides or proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, they may be subjected to amino acid sequence analysis, mass spectra analysis or amino acid compositional analysis according to known methods.

E. General Formulae and Exemplary Compositions of the Invention

The APC-targeted antigens of the invention can comprise one or more antigenic moieties, and likewise can comprise one or more targeting moieties. Moreover, these moieties can be functionally linked in several ways. For instance, if "T" represents a targeting moiety, and "Ag" represents an antigenic moiety, the APC-targeted antigens of the present invention may be organized as follows:

Ag - T;

T - Ag;

T_1 - Ag - T_2;

T_1 - $[Ag]_n$ - T_2 (wherein $[Ag]_n$ represents a multiplicity of antigens.

Examples of the general formulas set forth above include:

Ag - C5a agonist peptide;

IFNγ peptide - Ag;

IFNγ peptide - $[Ag]_n$ - C5a agonist peptide.

Other representative compositions of the invention include:

MUC1 Class I binding epitope - C5a agonist C-terminal peptide

Murine or human IFNγ peptide - MUC1 Class I binding epitope

Murine or human IFNγ peptide - MUC1 tandem repeat MUC1 Class I epitope - -C3dG peptide SAA-K-Ahx - C5a C-terminal peptide (Ahx = ϵamino hexanoic acid).

It will be appreciated by persons skilled in the art that the APC-targeted activating antigens of the invention may be adapted for inclusion of large or complex antigens. This may be accomplished, for example, by inclusion of a "spacer" (such as the K-Ahx spacer moiety in the exemplary compound above) between the antigen and the targeting moiety. Such chemical modifications are familiar to biochemists.

II. Uses of APC-Targeted Activating Antigens

The APC-targeted activating antigens of the present invention have broad potential for clinical applications in humans and animals. As discussed above, a significant impediment to the development of vaccines and other immunotherapeutic agents is the apparent inability of particular antigens to be readily taken up and processed by antigen presenting cells. The compositions of the invention facilitate the specific delivery of an antigen to a population of antigen presenting cells, whereupon the delivery mechanism (e.g., using as the targeting moiety a receptor ligand capable of transducing a biological signal) simultaneously activates the antigen presenting pathway. of the APC. Thus, the present invention enables development of vaccines and other immunotherapeutics that can specifically target any peptide antigen or other antigenic structure covalently attached to a ligand for a receptor present on an antigen presenting cell. It is believed that antigens linked to ligands that selectivity bind to and activate a particular population of APCs can not only generate an immune response, but can influence the nature of the immune response that is generated. Thus, immune responses that favor antibody, cellular, Th1

or Th2 responses, respectively, may be selectively generated. Vaccines may also be developed with an array of such targeting moieties thereby serving to target a selected antigen or antigens to several populations of APCs and simultaneously activate these and other cells involved in various immune modulatory pathways.

The ability to generate either antibody or cell mediated immune responses against different specific antigens has broad general applicability, and it is anticipated that the APC-targeted antigens of the invention will be extremely useful for these purposes. For example, antibody responses have been shown to be capable of protecting against different viral or bacterial infection, and antibodies are known to inactivate different toxins or toxic compounds that may affect the well being of humans or animals. Different cell mediated immune responses can provide protection against viral or other intracellular pathogens, and can play a role in some anti-tumor responses. It is believed that different antigen presenting cells and the context in which these cells are stimulated to present antigen (co-stimulation mediated by different ligand-receptor interactions) are important factors determining the nature of the above responses.

The targeted antigens of the present invention should find particular utility in the development of active specific immunotherapeutic agents (i.e., cancer "vaccines") based on cancer-associated antigens. For example, it has been hypothesized that induction of strong cell-mediated immune responses (involving Th1 cells and/or cytotoxic T lymphocytes) would provide the most effective protection against various forms of cancer. A vaccination strategy utilizing the APC-targeted antigens of the invention can be designed to induce this type of response. In this regard, it is known that stimulation with some cytokines (IL-12, IFNγ) can induce predominantly Th1 type responses over Th2 type

responses for certain antigens.

As a step toward developing anti-cancer vaccines for clinical use, the compositions of the invention can be used to advantage as research tools to further explore the effect of stimulating a certain population of APCs with a tumor antigen and determining the effect on an anti-tumor immune response. To this end, it should be noted that the present application exemplifies targeted antigens comprising an epitope of a particular tumor-specific antigen, Mucin-1.

Previous tumor vaccine formulations that aim to immunize patients with compounds that are identical to compounds already produced by tumors have proven to be of limited value, probably because tumors that progress have been selected for their lack of immunogenicity in their respective host (e.g., the host is tolerant to existing tumor antigens). Thus, one important challenge of producing effective tumor vaccines is generating reagents that counteract immunological tolerance to tumor-associated antigens. One purpose of the APC-targeted antigens described above is to induce in the immunized individual a response against their tumor that is similar to that seen in individuals undergoing allograft rejection. In other words, the goal is to induce an autoimmune reaction against the tumor that is capable of destroying the tumor. The immunological parameters that regulate tolerance to tumor antigens are not well understood; nonetheless the compositions described herein have the potential to counteract this tolerance and thus induce specific immune responses that mediate tumor rejection.

The targeted antigens of the present invention will also find broad utility in the production of antibodies for use as immunodiagnostic and immunotherapeutic agents. For immunodiagnostic purposes, antibodies are widely used in various quantitative and qualitative assays for the detection and measurement of

biological molecules associated with diseases or other pathological conditions. For reasons that often are not well understood, it is sometimes difficult to generate antibodies against certain biological molecules using conventional means. The compositions of the present invention provide an alternative means for inducing an animal to produce antibodies against a weakly-antigenic or non-antigenic substances. The utility of the compositions of the invention in this regard is shown clearly in Example 2, below, in connection with serum amyloid A. The appearance and abundance of this protein in the body is strongly correlated with systemic inflammatory stress, which is a condition that is very difficult to quantitate. It is believed that quantitative assays for SAA levels would be an excellent indicator of general, systemic inflammation; therefore it would be of benefit to generate antibodies against the protein in a non-human species. This protein has proved particularly recalcitrant to the generation of antibodies using conventional measures. As described in Example 2, a targeted antigen comprising SAA conjugated to a C5a peptide ligand produced a significant antibody response in mice injected with the conjugated molecule. In a similar fashion, targeted antigens comprising any weakly-antigenic or non-antigenic component of interest could be made and used to produce specific antibodies in laboratory animals, for use as immunodiagnostic reagents.

Antibodies for use as immunotherapeutic agents can also be generated using the compositions of the invention. As one example, there has been a great deal of recent interest in developing reagents capable of down-regulating or inhibiting the complement cascade to modulate local and systemic inflammatory responses. To this end, the C3a convertase, which is active early in the cascade, could provide an ideal target for complement inhibition. C3a convertase cleaves the peptide C3 into two components, C3a and C3b, and therefore must be able

to access the cleavage site on C3 in order to accomplish the result. Antibodies directed toward the C3a-C3b cleavage site are expected to be effective in blocking access of C3a convertase to the cleavage site, thereby inhibiting this early step in the complement cascade. Such antibodies may be generated using a targeted antigen of the invention comprising, as the antigenic moiety, the short peptide sequence comprising the C3a/C3b cleavage site. The sequence could then be conjugated to an appropriate targeting moiety, such as the C5a C-terminal decapeptide agonists exemplified herein. Thus, the compositions would be useful to generate an immunotherapeutic agent (e.g., an antibody that blocks the activity of C3a convertase) for treating an adverse inflammatory condition.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate the invention in greater detail. They are not intended to limit the invention in any way.

EXAMPLE 1

Evaluation of Mucin Epitope (MUC1/C5a agonist) Conjugate for Recruitment and Activation of Antigen Presenting Cells (APCs) and Stimulation of an Immune Response in Mice

The C5a receptor is present on numerous antigen presenting cells, including monocytes, macrophages, dendritic cells, and other cell types. In this example, a composite peptide comprising a mucin epitope (MUC1) functionally linked to a decapetide agonist analog of C5a corresponding to the C-terminal effector region of the natural factor was evaluated for its ability to activate the APCs thereby stimulating an immune response in mice. This evaluation is based on the known property of C5a receptors to internalize and recycle in the antigen presenting cell, thereby acting as ideal candidates for delivering antigens to and simultaneously activating

signals in the APCs. Because C5a receptors are particularly common on macrophages, monocytes and dendritic cells, it is believed that the use of a C5a agonist analog to bind C5a receptors will result in preferential activation of these APCs.

i. Abbreviations. Except where noted, the single letter designation for the amino acid residues is used: alanine is A; arginine is R; asparagine is N; aspartic acid is D; cystine is C; glutamine is Q; glutamic acid is E; glycine is G; histidine is H; isoleucine is I; leucine is L; lysine is K; methionine is M; phenylalanine is F; proline is P; serine is S; threonine is T; tryptophan is W; tyrosine is Y; and valine is V. Upper case letters represent the L-amino acid isomer and lower case the D-isomer.

ii. Peptide synthesis, Purification and Characterization. The following peptides were synthesized according to standard solid-phase methodologies on an Applied Biosystems (Foster City, CA) model 430 A peptide synthesizer and characterized as previously described (7):

(1) The antigenic "juxta-membrane" (JM) epitope of the human mucin-1 (MUC1), YKQGGFLGL (SEQ ID NO:6);

(2) The C5a C-terminal decapeptide agonist analog, YSFKPMPLaR (SEQ ID NO:1);

(3) The composite peptide YKQGGFLGLYSFKPMPLaR (SEQ ID NO:2), in which the JM epitope is positioned toward the amino terminus and the C5a peptide is positioned toward the carboxyl terminus; and

(4) The composite peptide YSFKPMPLaRKQGGFLGL (SEQ ID NO:5), in which the JM epitope of MUC1 is positioned toward the carboxyl terminus and the C5a analog is positioned toward the amino terminus.

Peptide 3 retains C5a biological activity, whereas peptide 4 does not because the biologically important carboxyl terminal end of the C5a analog is

blocked by the presence of the mucin epitope. As such, peptide 4 serves as a control to determine the importance of the C5a biological activity to the effectiveness of these peptides for immunization purposes.

Syntheses were performed on a 0.25 mmol scale on 0-hydroxymethylphenoxymethyl polystyrene (HMP) resins (0.88 meq/g substitution). N^{α}-amino groups were protected with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group. Side-chain functional groups were protected as follows: Arg (Pmc or 2,2,5,7,8-pentamethylchroman-6-sulfonyl); Asp (Ot-butyl ester); Cys, Gln & His (Trt or trityl); Lys (Boc or *t*-butyloxycarbonyl); Ser & Tyr (*t*-butyl). Synthesis was initiated by the *in situ* coupling of the C-terminal residue (N^{α}-Fmoc-L-Arg(Pmc)) to the HMP resin in the presence of excess N-N'-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) with 4-dimethylaminopyridine (DMAP) as a coupling catalyst. Peptide chain elongation was accomplished by repetitive Fmoc deprotection in 50% piperidine in NMP followed by residue coupling in the presence of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU).

Side-chain deprotection and cleavage from the resin were achieved in a single step acetolysis reaction by stirring the peptide-resin in a solution of 84% trifluoroacetic acid (TFA), 6% phenol, 2% ethanedithiol, 4% thioanisole, and 4% water for 1.5 hr at room temperature. Free peptide was precipitated from this solution by adding cold diethyl ether. The mixture was filtered through a scintered glass Buchner funnel (medium porosity) and the peptide/resin washed twice with cold ether to remove the thiol scavenger. The peptide was extracted by swirling the peptide/resin in the funnel with 20-30 ml aliquots of 10% acetic acid followed by filtration. The extraction aliquots were combined, frozen, and lyophilized to yield the powdered form of the crude peptide.

Peptides were purified by preparative and analytical reverse-phase HPLC on columns packed with C_{18}-bonded silica. The details of this procedure have been described by (4). All peptides were characterized by amino acid compositional analysis and fast atom bombardment mass spectrometry (FAB-MS).

iii. Animal Models. The strains of mice used for this example were inbred females 6 to 12 week old C57B16($H\text{-}2^b$) and Balb/c ($H\text{-}2^d$), which were obtained from Jackson labs (Bar Harbor, Maine). These two strains which differ in H-2 haplotypes, were used in this example to demonstrate that the observed antibody responses were not a result of the selection or creation of an unique immunogenic epitope characteristic of the sequence of the proteins of the MHC class I and class II molecules important for antigen processing in one mouse strain or another. The MUC1 peptide selected for these studies contained a motif that may bind to the $H\text{-}2K^b$ molecule of the C57B16 mice; therefore, a strain of mouse that lacked this class I molecule binding motif (Balb/c) was also studied to determine the relative contribution of the class I binding motif to the antigen presentation properties of these peptides.

iv. Immunization protocol. Preimmune sera were obtained from mice, which were subsequently immunized intraperitoneally with 100 μg of the indicated peptide with RIBI adjuvant (MPL+TDM+CWS) (Sigma Immunochemicals). Animals were boosted twice at two week intervals using the same injection procedure. Sera were obtained following three immunizations (at 6 weeks).

v. Analysis of serum antibody responses. For radioimmunoassay (RIA), anti-peptide antibodies were determined, before and at different time points after immunization, in 96-well microtiter plates (Dynatech Laboratories, Inc.). Plates were coated with 50 μl of a 100 μg/ml appropriate peptide in phosphate-buffered saline (PBS) pH 7.4 solution overnight at 4°C. The wells

were blocked by incubation with 5% dry milk in PBS pH 7.4 for at least two hours. Anti-peptide antibody titers were determined using serial dilutions of sera. The sera were diluted with PBS containing 0.05% Tween-20, pH 7.4 (washing buffer) and 50 μl of each dilution was incubated at 37°C for 1 hour. The wells were then drained, washed 4 times with PBS-Tween and 50 μl of ^{125}I-goat anti-mouse Ab (1 - 2 x 10^4 cpm/well) was added and incubated for 1 hr at 25° C. After washing, specific radioactivity was recorded in a gamma counter (1272 CliniGamma, LKB).

Anti-peptide antibody isotype titers were determined by enzyme-linked immunosorbent assay (ELISA) carried out in 96-well microtiter plates. The plates were coated with 100 μg/ml of appropriate peptide in PBS, pH 7.4, and incubated overnight. The wells were blocked with 5% dry milk in PBS pH 7.4 for at least two hours. Anti-peptide titers were determined using serial dilutions of sera as described above. After the plates were washed 4 times, 50 μl of a 1:100 dilution of rabbit anti-mouse IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM (Zymed) was added to each well and incubated at 25°C for 1 hour. The plates were washed 4 times with washing buffer and 50 μl of 1:500 goat anti-rabbit conjugated to peroxidase (Zymed) was incubated at 37°C for 1 hour. Again, the plates were washed 4 times with washing buffer and bound enzyme was detected by the addition of 50 μl 1 mg/ml p-nitrophenyl phosphate (Sigma) in 10% diethanolamine (Sigma) pH 9.4. The reaction was stopped by the addition of 50 μl of 0.5 M sodium hydroxide and absorbance values (A_{405}) were determined on Titertek Multiskan (Flow Laboratories, Irvine, Scotland).

vi. Experimental groups. Experimental groups were as follows:

Group A. mice immunized with peptide (1)

Group B. mice immunized with peptide (2)

Group C. mice immunized with peptide (1) plus peptide (2)

Group D. mice immunized with peptide (3)
Group E. mice immunized with peptide (4).

The results of the experimental protocols are set forth in Figures 1 and 2. As can be seen in the Figures, the mice in Groups A, B, C and E produced no appreciable increase in antibody response to inoculation with MUC1 epitope (Group A), C5a agonist peptide (Group B), MUC1 epitope combined with, but not conjugated to, C5a agonist peptide (Group C), or MUC1 epitope conjugated to the C5a agonist peptide at its C-terminus, rather than its N-terminus (thereby blocking C5a biological activity) (Group E). Only mice inoculated with the MUC1 epitope/C5a agonist peptide conjugate of the present invention (Group D) generated an appreciable antibody response. Furthermore, this stimulation was significant. It is clear from these results that inoculation with the conjugated MUC1 epitope/C5a agonist peptide was far more efficient in stimulating a general immune response (i.e., production of antibodies) than was inoculation with either peptide alone, or both peptides together, but not conjugated, or peptides conjugated in the opposite orientation.

There are several significant conclusions that can be drawn based on these results. The fact that both Balb/c and C57B16 mice showed antibody responses to peptide 3 suggests that the antigen presenting effect is not restricted by MHC haplotype. The fact that immune responses were not produced to peptide 4, or to mixtures of peptide 1 and 2, but that substantial responses were produced to peptide 3, suggest that the effect is mediated by the C5a moiety of the peptide and that the immune response results from the simultaneous delivery of antigen peptide and C5a mediated activation signals to antigen presenting cells.

The isotypes of the anti-peptide antibodies produced in the immunized mice were determined (Figure 3)

and were found to consist of IgM, IgG2a, and IgG2b. This suggests that the immunogenic peptide is producing T cell-dependent responses, which generally require antigen processing and presentation. Data presented in Figure 4 show that the antibody response to peptide 3 includes a high percentage of antibodies that are specific for the MUC1 epitope that was the antigen moiety of these studies.

EXAMPLE 2

Evaluation of Serum Amyloid A/C5a Peptide Conjugates for Recruitment and Activation of APCs and Stimulation of Immune Response in Rats

Serum amyloid A is an acute-phase stress response protein generated by the liver. Along with other acute phase proteins, SAA is secreted in response to systemic inflammatory stress as a protective measure. SAA is of interest because it appears to be an excellent indicator of general, systemic inflammation, which is a phenomenon that is very difficult to quantitate. Because serum levels of SAA have been observed to parallel the rise and fall of the systemic inflammatory response, quantitation of serum levels of this peptide would provide an effective means of assessing inflammation. One way to accomplish this is to develop antibodies against SAA that could be used for quantitation such as in an ELISA assay. However, SAA has been particularly recalcitrant to the generation of antibodies against it. In this example, an evaluation was made of the ability of SAA conjugated to a C5a C-terminal analog (as described in Example 1) to activate antigen producing cells and produce an antibody response in rats.

i. Production and preparation of proteins and peptides. The C-terminal C5a analog K-Ahx-YSFKPMPLaR (SEQ ID NO:8) (AhX is aminohexanoic acid, which is a linear aliphatic

spacer moiety) was produced as described in Example 1. The aliphatic spacer moiety was included to separate the critical receptor-binding C5a analog from the bulky protein to be attached to the amino terminus.

Serum amyloid A was conjugated to the C5a peptide analogs according to the following method. SAA (100 μg) was reacted with a 50-fold molar excess of a water soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodiide (EDC), in 200 μl of phosphate buffered saline, pH 7.5, at room temperature for 30 minutes. A 50-fold molar excess of the peptide (K-Ahx-YSFKPMPLaR) (SEQ ID NO:8) and a 100-fold molar excess of a base diisopropylethyl amine (DIEA) were added to this solution. Water was added to the solution to bring the reaction mixture to a volume of 400 μl. This solution was stirred overnight at room temperature and then lyophilized to a dry powder. The powder was diluted to the appropriate volume with water to generate the stock mixture used for inoculating the animals.

ii. Experimental protocols. Rats were injected intraperitoneally with an inoculant comprising the SAA/C5a peptide conjugates in phosphate-buffered saline with or without RIBI adjuvant. Booster injections were given two and five weeks after the initial injections. The rats were sacrificed seven weeks after the initial injection and anti-mucin antibody production was assessed from the serum titers, as described in Example 1.

Significant anti-SAA antibody was produced from both groups of rats, whether or not RIBI adjuvant was included in the inoculation. As visualized by gel electrophoresis and autoradiography of anti-SAA antibody eluted from the plate assays, it appeared that anti-SAA antibody titers were essentially equivalent, or slightly higher, in rats inoculated with SAA/C5a peptide conjugate in the absence of RIBI adjuvant as compared to the same

inoculation without the adjuvant. Thus, antigenic conjugates comprising the C5a peptide analog are useful for generating antibodies against large proteins, as well as against smaller peptide fragments, such as those described in Example 1. Moreover, the successful generation of anti-SAA antibodies utilizing this method is particularly promising for purposes of producing antibodies against weakly- or non-antigenic peptides or proteins.

EXAMPLE 3

Production and Characterization of Site-Directed Neutralizing Antibodies Specific for a Peptide κR(33-52) from the Predicted Amino-Terminal Region of the Human Kappa Receptor

Receptors for human opioid peptide hormones have been described on numerous cell types. The receptors for μ, κ, and δ ligands have recently been cloned from genomic and cDNA libraries derived from normal tissue and cell lines. Considerable homology exists among the μ, κ, and δ receptors, except for the N-terminal regions of the receptors. The N terminal region of the human kappa receptor (amino acid residues 1-100) is relatively hydrophilic and would be predicted to be exposed on the surface of the cell membrane. A 20 residue peptide [κR(33-52)], was chosen and used to raise a site directed peptide specific polyclonal antibody (5).

The method of production of a polyclonal antiserum in rabbits using the molecular adjuvant, C5a-agonist peptide conjugated to the κR epitope is set forth below. The binding specificity and biological activities of the resulting polycolonal antiserum raised to the predicted extracellular region of the human kappa receptor (κR) are also described below.

i. Construction of Targeted-Immunogen. A peptide construct consisting of the κR(33-52) (FPGWAEPDSNGSAGSEDAQL) (SEQ ID NO:9) covalently attached to the N-terminal end of a conformationally biased, C5a

complement fragment agonist analogue peptide (YSFKPMPLaR) (SEQ ID NO:1) was synthesized according to the methods in Example 1 and as previously reported (7).

ii. Preparation of anti-κR(33-52) Antiserum and Peptide-Specific ELISA. Rabbits were immunized s.c. with 500 μg of FPGWAEPDSNGSAGSEDAQLYSFKPMLaR construct (SEQ ID NO:10) in complete Freund's adjuvant (GIBCO, Grand Island, NY) on day O followed by booster injections on days 30 and 60 in incomplete Freund's adjuvant. Serum was collected starting 75 days after the initial immunization.

The presence of anti-peptide antibody was determined by using a peptide specific ELISA utilizing the free κR(33-52) peptide as previously described (8). Anti-κR(33-52) and normal rabbit γ-globulin (RGG) were purified by protein A Sepharose chromatography (Sigma) (8) prior to use.

iii. Cells and culture conditions. The neuroblastoma cell SK-N-SH (HTB 11), ductal breast cell carcinoma T47D (HTB 133) , Jurkat T cell leukemia, (TIB 152), U937 histolytic lymphoma (CRL1593), THP 1 human monocyte (TIB 202), EBV-transformed B cells SKW 6.4 (TIB 215) and CESS (TIB 190) (American Type Culture Collection, Rockville, MD) were cultured in DMEM or RPMI 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 1 mM L-glutamine, 2 mM Na pyruvate, 50 U penicillin and 50 μg/ml streptomycin. The human neuronal precursor cells NT2 (Stratagene, La Jolla, CA) were cultured in Opti-MEM (Gibco) supplemented as above. All cultures were incubated at 37° C in a humidified chamber with 7.5% CO_2.

Peripheral blood derived mononuclear cells were obtained from healthy male and female volunteers, isolated by Ficoll-Hypaque(tm) density gradient centrifugation and enriched for macrophage by adherence to plastic.

iv. Flow Cytometry. Single-color flow cytometry analysis of cells (1 X 10^6) in PBS containing 1% bovine calf serum and 0.1% sodium azide (staining buffer) were preincubated 30 minutes at 4° C in the presence of 20% normal human serum. The cells were washed and incubated with anti-κR(33-52) or RGG for 30 minutes at 4° C, washed and labeled with PI-conjugated donkey (Fab')2 fragments of antirabbit IgG (Zymed, S. San Francisco, CA) for 30 minutes at 4° C (8). For dual color analysis FITC-conjugated anti-CD3 or anti-CD14 (Pharmingen, San Diego, CA) were also included in the second step. Cells (1 x 10^4) were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) and data were analyzed with the Cell Quest software as previously described (8).

v. Measurement of cell proliferation. Peripheral blood mononuclear cell (PBMC) were pulsed on day 2 of culture with ^{3}H-thymidine and 18 hours later the cells harvested on glass fiber filters and processed for scintillation counting. Experiments were performed three times and each sample done in triplicate.

vi. Measurement of IgG Secretion. Relative IgG levels in culture supernatants were determined by indirect ELISA as previously described (9). Supernatants from PBMC cultures were collected after 10 days and assayed for the presence of IgG. Numbers represent the mean CPM +/- SD from triplicate samples. Experiments were performed at least three times.

vii. Characterization of Anti-κR Peptide Antiserum. Serum from rabbits immunized with the κR(33-52)YSFPMPLaR construct (SEQ ID NO:10) and normal rabbit serum were assayed for the ability to recognize plate bound κR(33-52) (SEQ ID NO:9) in ELISA. The results show that serum from rabbits immunized with the κR(33-52)YSFPMPLaR construct (SEQ ID NO:10) bound free κR(33-52) peptide (SEQ ID NO:9) in a dose dependent fashion. The titer was approximately

10^5. In contrast, serum from unimmunized rabbits failed to bind this peptide. Serum samples from immunized and unimmunized rabbits were subjected to protein A-Sepharose chromatography and the column eluates were assessed for κR(33-52) (SEQ ID NO:9) specific antibody. The results indicate that protein A-purified antibody derived from rabbits immunized with the κR(33-52)YSFPMPLaR construct (SEQ ID NO:10) binding to free κR(33-52) (SEQ ID NO:9) was detectable at antibody concentrations less than 0.1 ng/ml. In contrast, RGG failed to bind the free peptide. The results from multiple bleedings indicated that the ED_{50} titer ranged between 1-10 ng/ml. These results indicate that rabbits immunized with κR(33-52)YSFPMPLaR (SEQ ID NO:10) contained high titer, κR(33-52) peptide specific antibody.

viii. Binding of anti-R (33-52) antibody to cells expressing human κR. To determine whether the polyclonal anti-κR(33-52) antibodies bound to cells expressing the κR, a variety of mononuclear cell lines and normal human mononuclear cells were first assayed for the presence of the κ receptor specific mRNA by RT-PCR. RNA samples isolated from neuronal cell lines NT2, U937, Jurkat, T47D, normal human PBMC, and enriched human macrophage were subjected to RT-PCR analysis with 5′ sense and 3′ antisense primers specific for the 3′ region of the cloned κR and B-actin. All of the cell lines or cell fractions, except for the T47D cell line, were positive for the κ-receptor specific PCR product, as expected based on the primer sequences used (5).

Experiments were performed to determine whether anti-κR(33-52) bound to cells expressing κR specific mRNA. The results of single color flow cytometric analysis for several cell lines are shown in Table 2. Flow cytometric measurements were conducted with human cell lines representative of macrophage (U937), T

lymphocytes (Jurkat), and B lymphocytes (SKW 6.4 and CESS). The results indicate that anti-κR(33-52) bound all three cell types. Anti-κR(33-52) bound to U937 cells to the greatest extent (MFI=231) compared to normal RGG (MFI=38). As used herein MFI refers to mean fluorescence intensity. Comparison of anti-κR(33-52) and RGG binding to the Jurkat line indicated approximately a 3-fold shift in MFI (MFI=18 vs. MFI=6). Similar results were obtained with the two B lymphocyte-like cell lines (SKW 6.4 and CESS). Comparison of anti-κR(33-52) and RGG binding to the SKW 6.4 line indicated approximately a 3-fold shift in MFI (MFI=19 vs. MFI=6). The neuronal cell line was also specifically bound by the anti-κR(33-52) as indicated by a 3-fold shift in the MFI over the RGG. Finally, based on the lack of expression of κR-specific mRNA from the human breast carcinoma cell line (T47D), this cell line was assessed for its ability to bind to anti-κR(33-52) by flow cytometric analysis. The lack of a κR expression on T47D cells was confirmed by the fact that anti-κR(33-52) and RGG bound to these cells in an almost identical fashion. As a positive control, anti-κR(33-52) and RGG were assessed for their ability to bind to an additional human macrophage-like cell line (THP 1). Comparison of anti-κR(33-52) and RGG binding to this cell line resulted in a significant shift in MFI (MFI=190 vs. MFI=8). These results confirm the specificity of anti-κR(33-52) for the human κR.

Table 1

Selected cell type binding of anti-κR(33-52) antibodies produced in rabbits immunized with C5a-agonist peptide conjugated to the κR(33-52) sequence as assessed by single channel color flow cytometric analysis.

		Mean Channel Intensity	
Cell Line	Cell Type	RGG	anti-κR Ab
NT2	Neuronal	9	19
U937	Macrophage	38	231
Jurkat	T-lymphocyte	6	18
SKW 6.4	B-lymphocyte	6	19
CESS	"	<10	>10
Controls			
T47D (negative)	Human Breast Carcinoma	~3	~3
THP1 (positive)	Macrophage	8	190

Analysis of intact human PBMC indicated that these cells express mRNA for a "κ-like" R (5). Dual color flow cytometric analysis was utilized to assay for the binding of anti-κR(33-52) to normal human macrophage (CD14+) and T lymphocytes (CD3+). It was observed that both macrophage and T lymphocytes bound anti-κR(33-52) antibody. Anti-κR(33-52) and RGG were assessed for binding to CD14+ PBMC. The results indicate that anti-κR(33-52) bound CD14+ cells with a 15-fold increase compared to normal RGG (MFI=320 vs. MFI=21). Anti-κR(33-52) was also found to bind CD3+ cells (MFI=19 vs. RGG MFI=3) albeit less than CD14+ cells. These results indicate that anti-κR(33-52) binds normal PBMC-derived mononuclear cells as well as mononuclear cell lines, which express κR-specific mRNA.

ix. Neutralization of U50,488H-mediated suppression of lymphocyte proliferation by anti-κR(32-52) antibody in vitro. The results of published studies have shown that

opioid peptide-induced regulation of *in vitro* immune responses can occur via specific receptor-ligand interactions. More specifically, it has been shown that the κR-selective agonist U50,488H is capable of suppressing SAC-induced lymphocyte proliferation by human PBMC cultures (6). The inhibition of lymphocyte activation by U50,488H has also been shown to be reversed by the κR-selective antagonist nor-BNI. To determine whether anti-κR(33-52) was capable of acting as an κR selective antagonist and neutralizing U50,488H-mediated suppression, PBMC cultures were preincubated with various concentrations of protein A purified anti-κR(32-52) prior the addition of SAC and U50,488H. U50,488H suppresses SAC-induced lymphocyte proliferation in a dose dependent fashion (5). Maximal suppression was obtained when U50,488H was used at a concentration of 10^{-6} M. PBMC cultures were preincubated with various concentrations of anti-κR(33-52) (1-50 μg/ml), followed by the addition of U50,488H plus SAC, and proliferation measured on day 3 of culture. Anti-κR(33-55) was found to neutralize U50,488H-mediated suppression of SAC-induced lymphocyte proliferation in a dose dependent fashion. In contrast, identical concentrations of normal RGG failed to inhibit κR selective agonist mediated immunosuppression.

Since SAC has been shown to induce both T and B lymphocyte proliferation, similar experiments were conducted with the T cell mitogen PHA. Anti-κR(33-52) was also able to neutralize the ability of U50,488H to suppress mitogen-induced T cell proliferation. U50,488H (10^{-6} M) suppressed PHA-induced T cell proliferation by 85%. This suppression was reversed by preincubating the cells with anti-κR(33-52). Preincubation of PBMC with normal RGG failed to block U50,488H-mediated suppression of T cell proliferation.

Anti-κR(33-52) does not appear to directly modulate lymphocyte proliferation. The co-culture of PBMC with anti-κR(33-52), in the absence of mitogen, failed to

stimulate the cells above the media control. Moreover, the combination of anti-κR(33-52) and PHA or SAC did not result in increased cell proliferation compared to PBMC cultures receiving mitogen only.

x. Neutralization of U50,488N-mediated suppression of IgG synthesis by anti-κR(32-52) antibody in vitro. In addition to lymphocyte proliferation, U50,488H is a potent inhibitor of SAC-induced IgG synthesis in human PBMC cultures (6). To determine whether anti-κR(32-52) was capable of neutralizing the suppression of IgG synthesis, PBMC were preincubated with anti-κR(32-52) followed by the addition of U50,488H and SAC, and IgG levels measured on day 10. Results indicate that U50,488H at 10^{-8} M and 10^{-7} M inhibited IgG synthesis by 67% and 85% respectively (5). The inclusion of anti-κR(32-52) in culture was found to neutralize suppression of SAC induced IgG synthesis in a dose dependent manner. In contrast, similar concentrations of normal RGG failed to neutralize the observed suppression.

To assess the specificity of anti-κR(32-52) antibody, PBMC were incubated with specific antibody or RGG followed by co-culture with U50,488H or the μ receptor selective agonist (DAGO) and IgG production measured by ELISA. The results indicate that, whereas, anti-κR(32-52) neutralized U50,488H-mediated inhibition of SAC-induced IgG synthesis, anti-κR(32-52) was unable to neutralize DAGO-mediated suppression of IgG synthesis.

These results indicate that in addition to binding lymphocytes and macrophage, anti-κR(32-52) is capable of neutralizing the ability of a κR selective agonist (U50,488H), but not a μR selective agonist (DAGO). Additionally the antibody demonstrated significant inhibition of both lymphocyte proliferation and differentiation to antibody synthesis. These results further demonstrate the specificity of anti-κR(33-52) for

the human kappa receptor.

As can be seen from the antibody binding data presented above, the site directed polycolonal antibodies raised in rabbits using the C5a-agonist form of the molecular adjuvant conjugated to the κ receptor sequence were capable of binding to normal human cells and cell lines expressing mRNA specific for the human κ receptor. Flow cytometric analysis of a neuronal cell line (NT2), normal blood-derived CD14+ monocytes, monocyte-like cell lines (U937 and THP1), normal blood derived CD3+ T cells and a T cell line (Jurkat), and human B cell lines (SKW6.4 and CESS) revealed that the cells were all bound by anti-κR(33-52) in a specific manner. The anti-κR(33-52) did not bind to a cell line determined not to express mRNA for the human κ receptor.

Anti-κR(32-52) was found to specifically neutralize κR-selective agonist (U50,488H)-mediated inhibition of lymphocyte activation. The antiserum was found to neutralize, in a dose dependent manner, U50,488H-mediated inhibition of: 1) SAC-induced lymphocyte proliferation; 2) PHA-induced lymphocyte proliferation and; 3) SAC-induced IgG synthesis. In contrast, DAGO-mediated suppression of SAC-induced IgG production was not affected by anti-κR(32-52). These results suggest that this site directed polyclonal antiserum specifically interacts with the human κR on PBMC. The results presented indicate that polyclonal anti-κR(32-52) antibodies interact with the exposed N-terminal region of the κR. While this antiserum effectively blocked U50,488H -mediated lymphocyte activation, it did not activate macrophage or lymphocytes.

While anti-κ opioid receptor antibodies are exemplified above, conjugation of C5a agonist peptide to peptides corresponding to μ and Δ specific peptides has resulted in the successful generation of specific antibodies to the μ and Δ epitopes.

EXAMPLE 4.

Comparison of Immunogenicity of Epitope-C5a agonist constructs with epitope-KLH conjugates.

The following experiment was performed in order to compare the potency of the molecular adjuvant of the present invention with a widely used method for enhancing the immune response to peptide epitopes. The objective was a direct comparison of the response to a construct of MUC1 epitope-C5a agonist and the same epitope conjugated to keyhole limpet hemocyanin (KLH) in mice. The results are summarized in Table 2.

Table 2

MUC1 Specific Ab Isotype Titers Produced with Different Immunogens.

	Ab Isotypes and Titers[a]					
	IgA	IgG1	IgG2a	IgG2b	IgG3	IgM
YKQGGFLGLYSFKPMPLaR[b] (SEQ ID NO:2)	0	0	1260 (5/5)	1780 (5/5)	0	6310 (5/5)
YKQGGFLGL-KLH[c] (SEQ ID NO:6-KLH)	0	100 (2/5)	0	0	0	5010 (4/5)

[a] Sera were screened against MUC1 peptide and mean titer values of responders are shown. Parentheses indicate the number of responders. Ab titer is defined as the sera dilution within the linear range at which specific reactivity is lost.

[b] Five C57BL6 mice were immunized and boosted with YKQGGFLGLYSFKPMPLaR (SEQ ID NO:2) and sera were obtained as indicated in the Material and Methods section. Standard error of responder titer values was less than 32% for all isotypes.

[c] Five C57BL6 mice were immunized and boosted with YKQGGFLGL-KLH (SEQ ID NO:6 conjugated to KLH) and sera were obtained as indicated in the Materials and Methods section. Standard error of responder titer values was less then 25% for IgM and less than 40% for IgG1.

A similar experiment was performed in rabbits. The immunogens used in rabbits were the κ- and μ- opioid

receptor epitopes, FPGWAEPDSNGSAGSEDAQL (SEQ ID NO:9) and GDLSDPCGNRTNLGGRDSL (SEQ ID No:11), respectively. The serum antibody titer and antibody subtypes produced in rabbits injected with the two compositions containing the different immunogens were compared.

i. Peptide conjugates. In one instance the epitopes were conjugated to KLH via a lysine residue added synthetically to the N-terminus of the epitope along with an alanine residue which acted as a spacer. In this experiment, glutaraldehyde was used to effect conjugation. In the another case, the epitopes were linked to the N-terminal end of the C5a agonist YSFKPMPLaR (SEQ ID NO:1) using the solid phase peptide synthetic methodologies described above in example 1.

ii. Immunization protocol for rabbits. Rabbits were immunized s.c. with 500 μg of either the epitope-KLH or the epitope-YSFKPMPLaR (epitope-SEQ ID NO:1) constructs in compete Freund's adjuvant (GIBCO, Grand Island, NY). Booster injections were administered on days 30 and 60 in incomplete Freund's adjuvant. Serum was collected starting at day 60 post-immunization.

iii. Antibody determination. The presence of rabbit IgG specific for the peptide epitopes was determined by ELISA as previously described (8).

Rabbits immunized with the epitope-C5a agonist generated high titer IgG Abs specific for the opioid receptor peptide epitopes. The rabbits immunized with the opioid receptor epitopes conjugated to the carrier protein KLH also produced high titer antibodies specific epitopes to which they were injected. These results demonstrate that the decapeptide C5a-agonist was as effective as the large molecular weight protein, KLH, conjugated to the epitope at inducing specific anti-peptide antibodies in non-rodent species.

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While certain preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications

may be made to the invention without departing from the scope and spirit thereof as set forth in the following claims.

5

WHAT IS CLAIMED IS:

1. A molecular adjuvant for enhancing an immune response to an immunogen comprising:
a targeting ligand having binding affinity for a characteristic determinant of an antigen presenting cell, said targeting ligand being functionally linked to said immunogen, whereby binding of said molecular adjuvant to said antigen presenting cell determinant activates said antigen presenting cell, effecting delivery of said immunogen to an antigen presenting pathway of said antigen presenting cell.

2. A molecular adjuvant as claimed in claim 1, wherein said targeting ligand binds specifically to a determinant comprising an immunomodulatory receptor of said antigen presenting cell.

3. A molecular adjuvant as claimed in claim 2, wherein said targeting ligand binds specifically to a receptor selected from the group consisting of C5a receptor, IFN-gamma receptor, CD21 (C3d) receptor, CD64 (FcgRI) receptor, and CD23 (FceRII) receptor.

4. A molecular adjuvant as claimed in claim 3, wherein said targeting ligand binds specifically to a C5a receptor and is selected from the group consisting of C5a and a peptide agonist analog of C5a comprising the C-terminal ten residues of C5a.

5. A molecular adjuvant as claimed in claim 4, wherein said targeting ligand is a peptide comprising the sequence YSFKPMPLaR, which is SEQ ID NO:1.

6. A molecular adjuvant as claimed in claim 1, comprising a targeting ligand and an immunogen

having the sequence YKQGGFLGLYSFKPMPLaR.

7. A molecular adjuvant as claimed in claim 1, wherein said targeting moiety and said immunogen are linked by a spacer moiety.

8. A molecular adjuvant as claimed in claim 3, wherein said targeting ligand binds specifically to an IFN-gamma receptor and is selected from the group consisting of IFN-gamma and a peptide analog of IFN-gamma comprising the N-terminal 39 residues of INF-gamma.

9. A molecular adjuvant as claimed in claim 8, wherein said targeting ligand is a peptide comprising a sequence selected from the group consisting of

HGTVIESLESLNNYFNFFGIDVEEKSLFLDIWRNWQKDG, which is Sequence I.D. No. 3; and

QDPYVKEAENLKKYFNAGHSDVADNGTLFGIKNWKEE, which is Sequence I.D. No. 4.

10. A molecular adjuvant as claimed in claim 1, wherein said immunogen comprises at least one substance selected from the group consisting of peptides, glycopeptides, phosphopeptides, lipopeptides, proteins, glycoproteins, phosphoproteins, lipoproteins, carbohydrates, nucleic acids and lipids.

11. A molecular adjuvant as claimed in claim 10, wherein said immunogen comprises a peptide.

12. A molecular adjuvant as claimed in claim 10, wherein said peptide comprises an epitope of human mucin-1.

13. A molecular adjuvant as claimed in claim 10, wherein said immunogen comprises a protein.

14. A molecular adjuvant as claimed in claim 13, wherein said protein comprises serum amyloid A (SAA).

15. A molecular adjuvant as claimed in claim 14 having the formula SAA-K-Ahx-YSFKPMPLaR, which is SAA-conjugated SEQ ID NO:8.

16. A molecular adjuvant as claimed in claim 1, wherein said immunogen comprises a tumor-specific antigen.

17. A composition for enhancing an immune response to an immunogen in a subject in which said enhanced immune response is desired, said composition comprising the molecular adjuvant of claim 1 in a biologically compatible medium.

18. A method for activating an antigen presenting cell for inducing an enhanced immune response to an immunogen, said immunogen being delivered to the antigen presenting pathway of said antigen presenting cell, said method comprising binding to a characteristic surface determinant of said antigen presenting cell a molecular adjuvant as claimed in claim 1.

19. A method as claimed in claim 18, wherein binding of said molecular adjuvant to said antigen presenting cell induces a humoral immune response.

20. A method as claimed in claim 18, wherein binding of said molecular adjuvant to said antigen presenting cell induces a cellular immune

response.

21. A method as claimed in claim 18, wherein said antigen presenting cell is selected from the group consisting of monocytes, dendritic cells, macrophages and B cells.

22. A method for eliciting an antigen presenting cell-mediated immune response in a host susceptible to infection by an antigen containing disease causing agent, said method comprising administering to said individual a molecular adjuvant, as claimed in claim 1, wherein said immunogen comprises the antigen of said disease causing agent, in an amount effective for eliciting said immune response.

23. A method for eliciting an immune response to a tumor-associated antigen, said method comprising administering to a host having a tumor expressing said tumor-associated antigen a molecular adjuvant as claimed in claim 1, wherein said immunogen comprises said tumor-associated antigen, in an amount effective for eliciting said immune response.

24. A method for the production of antibodies to an immunogen, comprising:

a) immunizing an animal with an immunogenic effective amount of the molecular adjuvant of claim 1;

b) isolating antibodies form sera of said animal; and

c) recovering said isolated antibodies.

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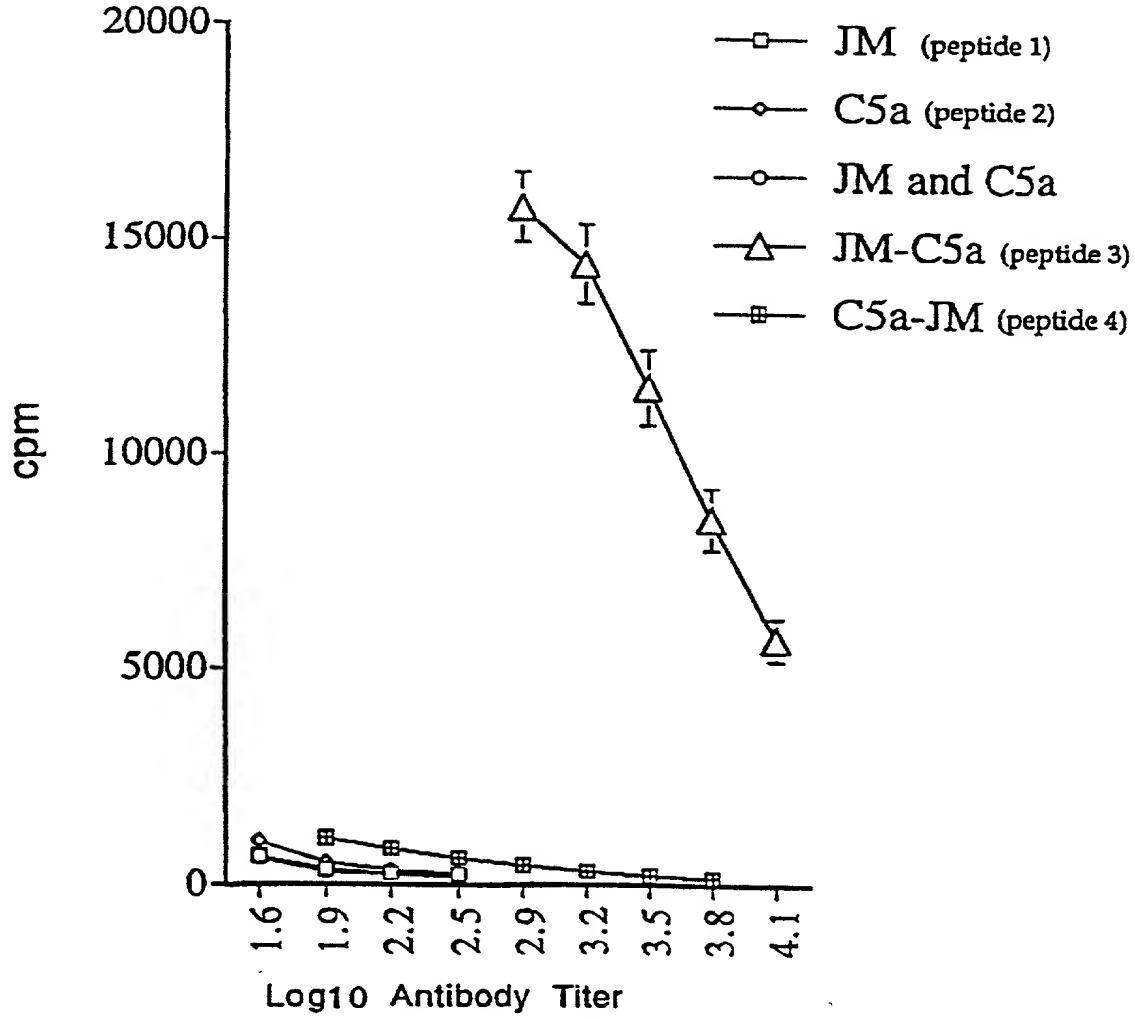

Figure 1

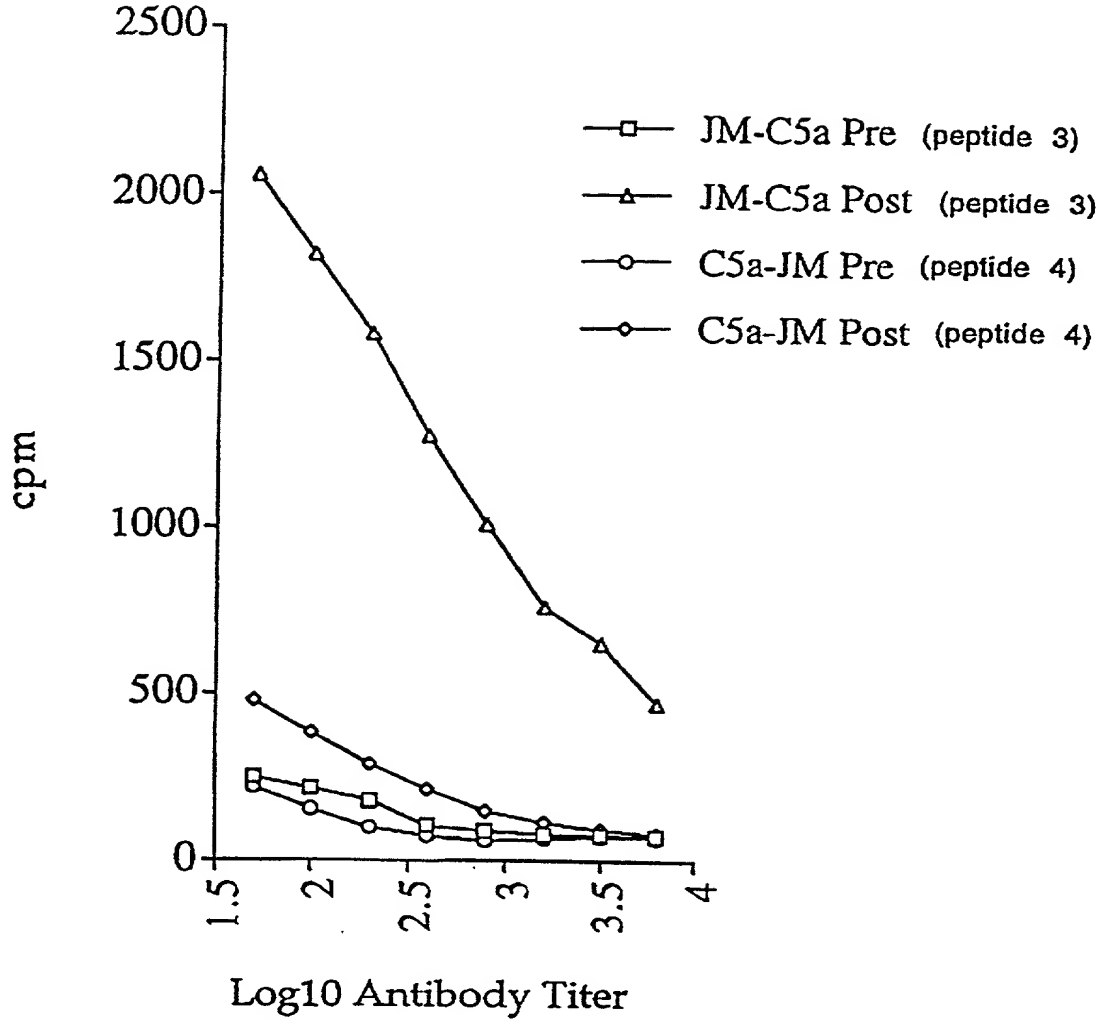

Figure 2

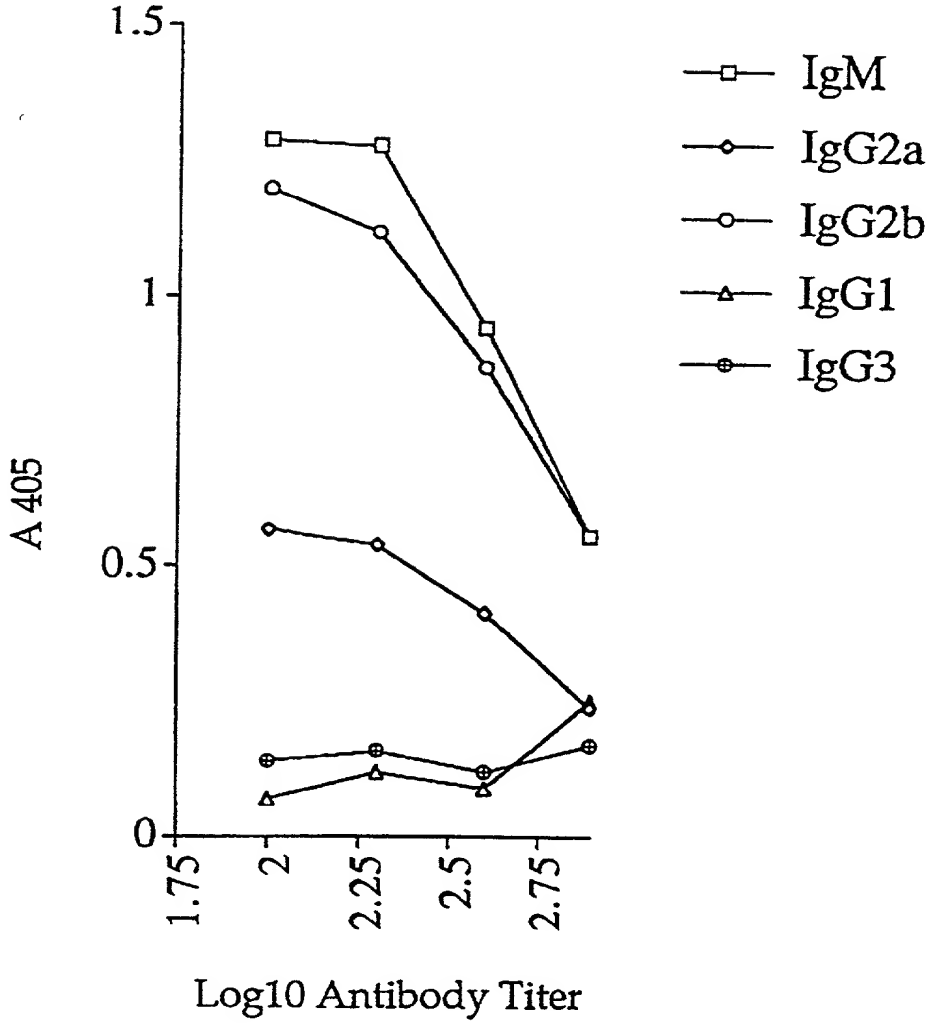

Figure 3

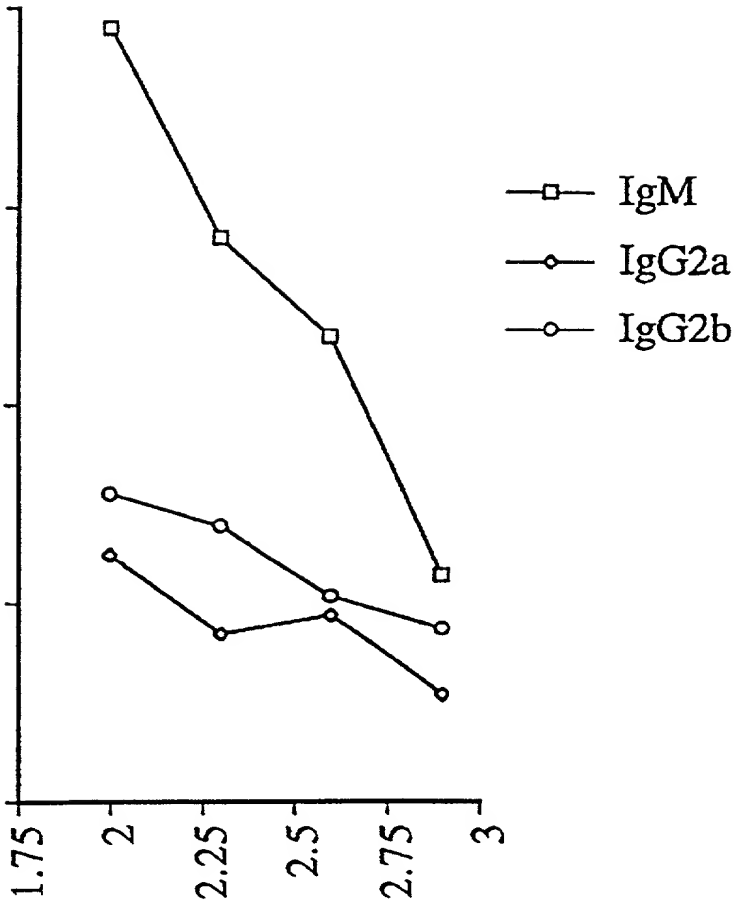

Figure 4

DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled:

the specification of which [check one(s) applicable]
X was filed April 17, 1998 as U.S. Application No. 09/051,685
___ and was amended by Amendment filed _____ (if applicable); [or];
___ is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and

that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56(a) [37CFR§1.56(a)].

CLAIM UNDER 35 USC §119(e): I hereby claim the benefit under 35 USC §119(e) of any United States provisional applications listed below:

Provisional Application No.	Filing Date Day/Mo/Year
60/005,727	20 October, 1995

POWER OF ATTORNEY: As inventor, I hereby appoint **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **Janet E. Reed, Ph.D., Reg. No. 36,252.**

POWER TO INSPECT: I hereby give **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.

SEND CORRESPONDENCE TO: **CUSTOMER NUMBER 000110.**

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Applicant or Patentee: Sam D. Sanderson, Michael A. Hollingsworth and Richard A. Tempero

Application or Patent No.: Not Yet Assigned

Filed or Issued: Concurrently Herewith

For: COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS

VERIFIED STATEMENT (DECLARATION) SUPPORTING ANOTHER'S CLAIM FOR SMALL ENTITY STATUS [37 CFR §1.9(f) AND §1.27(d)] - NONPROFIT ORGANIZATION

I hereby declare that I am making this verified statement to support a claim by the above-identified applicant or patentee for small entity status for purposes of paying reduced fees with regard to the above-identified invention described in

[X] the specification filed herewith
[ ] U.S. Provisional Application No. ____________________, filed ____________________
[ ] U.S. Patent No. ____________________, issued ______________

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

FULL NAME OF ORGANIZATION:

BOARD OF REGENTS OF THE
UNIVERSITY OF NEBRASKA

ADDRESS OF ORGANIZATION:

Regents Hall
3835 Holdrege Street
Lincoln, NE

TYPE OF ORGANIZATION

[X] University or other institution of Higher education
[ ] Tax exempt under U.S. Internal Revenue Code (26 USC§§501(a) and
[ ] Nonprofit scientific or educational under statute of state of U.S.A.
Name of State:
Citation of Statute:
[ ] Would qualify as tax exempt under U.S. IRC if located in U.S.A.
[ ] Would qualify as nonprofit scientific or education under statute of state of U.S.A if located in U.S.A.
Name of State:
Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR §1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States code to the above-identified invention.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization known to have rights to the invention is listed below* and the organization knows of no rights to the invention being held by any person, other than the inventor, wh could not qualify as an independent inventor under 37 CFR §1.9(c) if that person had made the invention, or by any concern which woul not qualify as a small business concern under 37 CFR §1.9(d) or by a nonprofit organization under 37 CFR §1.9(e).

FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR §1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR §1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so mad punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Linda S. Johnson

Title in Organization: Registered Patent Agent, Department of Technology Development, University of Nebraska Medical Center

Address: 600 S. 42nd Street, Box 986099, Omaha, NE 68198-6099

Signature: Linda S. Johnson   Date: 4/17/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: Sam D. Sanderson, Michael A. Hollingsworth and Richard A. Tempero

Application or Patent No.: 09/051,685

Filed or Issued: April 17, 1998

For: COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS [37 CFR §1.9(f) AND §1.27(b)] - INDEPENDENT INVENTOR(S)

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR §1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled described in

[ ] the specification filed herewith
[X] U.S. Application No. 09/051,685, filed April 17, 1998
[ ] U.S. Patent No. _____, filed _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR §1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR §1.9(d) or a nonprofit organization under 37 CFR §1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

[ ] no such person, concern, or organization
[X] person, concerns or organizations listed below*

FULL NAME: BOARD OF REGENTS OF THE UNIVERSITY OF NEBRASKA
ADDRESS: Regents Hall, 3835 Holdrege Street, Lincoln, NE 68198

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [X] NONPROFIT ORGANIZATION

FULL NAME:
ADDRESS:

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR §1.27)

I acknowledge the duty to file, in this application or patent, notification of any changes in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR §1.28(b)).

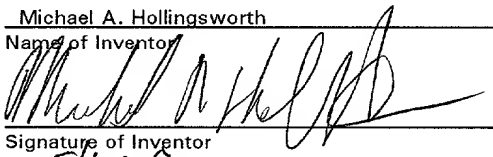
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Sam D. Sanderson
Name of Inventor

Signature of Inventor

5-13-98
Date

Michael A. Hollingsworth
Name of Inventor



Signature of Inventor

5/13/98
Date

Richard A. Tempero
Name of Inventor

TEMPERO
Signature of Inventor

5-13-98
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of: Sam D. Sanderson, et al.

10 Rec'd PCT/PTO 19 AUG 1998

Application No.: 09/051,685

Filed: April 17, 1998

For: **COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS**

Certificate of Mailing Under 37 C.F.R. §1.8(a)

I hereby certify that this correspondence is being deposited on August 17, 1998 with the United States Postal Service as first-class mail in an envelope properly addressed to COMMISSIONER OF PATENTS AND TRADEMARKS, Washington, DC 20231.

August 17, 1998
Date of Certificate

Janet E. Reed, Ph.D.
PTO Registration No. 36,252

United States Department of Commerce
Patent and Trademark Office
Assistant Commissioner For Patents
Box PCT
Washington, D.C. 20231

Dear Sir:

08/24/1998 PVOLPE 00000133 09051685
01 FC:254 65.00 OP

In response to the NOTICE OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) dated July 24, 1998, a copy of which is enclosed herewith, we are completing the above-identified U.S. Patent Application of: Inventor(s): Sam D. Sanderson, et al.

For: COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS
The complete application includes the following:

50	Pages of Specification	Filed April 17, 1998
24	Number of Claims	Filed April 17, 1998
1	Page of Abstract	Filed April 17, 1998
4	Sheet(s) of Drawings	Filed April 17, 1998
2	Pages of Declaration and Power of Attorney	Filed concurrently herewith
1	Number of Verified Statement(s) under 37 CFR §§1.9 and 1.27 to establish Small Entity Status	Filed concurrently herewith
0	Assignment Form, including Recordation Form Cover Sheet	Filed
0	Preliminary Amendment	Filed

The Fee has been calculated as follows:

CLAIMS AS FILED			
FOR	CLAIMS (AFTER AMDT.)	////////// ////////// //////////	NUMBER EXTRA
BASIC FEE			
FFECTIVE TOTAL CLAIMS	24	-20	= 4
INDEPENDENT CLAIMS	2	-3	= 0
MULTIPLE DEPENDENT CLAIMS			
SURCHARGE FOR LATE FILING			
EXTENSION FEE (month) $			

SMALL ENTITY	
RATE	FEE
395	0
11	0
41	0
125	0
65	65
	0
TOTAL	65

OTHER THAN A SMALL ENTITY	
RATE	FEE
790	
22	
82	
270	
130	
TOTAL	

[ ] Assignment Recording Fee to be charged to Deposit Account No. 04-1406.
[X] A check in the amount of $ 65.00 is enclosed. In the event the check is improper, or the fee calculation is in error, the Commissioner is authorized to charge any underpayment or credit any overpayment to the account of the undersigned attorneys, Account No 04-1406.
[X] A duplicate copy of this sheet is enclosed.
[X] Please address all communications to the following Correspondence Address:

Janet E. Reed, Ph.D.
DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.
1601 Market Street, Suite 720
Philadelphia, Pennsylvania 19103-2307

Telephone (215) 563-4100
Facsimile (215) 563-4044
Direct all inquiries to the undersigned attorney.

Respectfully submitted,
DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By: _____
Janet E. Reed, Ph.D.
PTO Registration No. 36,252